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Approaches for Enhancing *in vitro* Production of Plant Secondary Metabolites

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Introduction

Plants synthesize an immense and varied variety of organic compounds, most of which are not specifically involved in development and growth. These com pounds are of two types popularly referred to as primary and secondary metabolites. The primary metabolites are directly involved in energy transducing pathways and must for normal growth, development, and reproduction. The metabolites such as carbohydrates, lipids, amino acids (Fig. 1), vitamins and proline (Fig. 2) are the common examples of primary metabolites. The chemical substances produced in the cells of plant during different metabolic process are known as secondary metabolite. The chemical structures are the basis of secondary metabolite classification. Some chemists have been interested in these new phytochemicals since the 1850s, and have investigated their chemical characteristics. The secondary metabolites are not directly involved in normal growth, development and reproduction. In order to communicate with the surrounding biotic and abiotic environment, secondary metabolites are organic compounds formed by plants. The natural products like Rosin (abietic acid) and vanillin (phenolic acids), salicylic acid, and caffeic acids are known as secondary metabolites. The classification of plant natural products is based on their biosynthetic origin and divided into groups: the alkaloids, the terpenoids, the sapaonins, and phenolic compounds. The alkaloids with one or more nitrogen are primarily biosynthesized from amino acids. Approximately 8000 phenolic compounds are produced by two pathways that are not distinguishable on the basis of precursor molecules, chemical structures, or biosynthetic sources, either by the shikimic acid pathway or the malonate/acetate pathway. For example, kaurenoic acid (Fig. 3) and abietic acid (found in *Asafetida*) are formed by similar sequence of related enzymatic reaction. Different biological activities are carried out by most secondary metabolites and few of them are used as pharmaceuticals, agrochemicals, flavours, fragrances, colours, and textile. The secondary metabolites are largely obtained from plant cultivation in the field, but this platitudinous approach has different drawbacks, such as tissue- and age- specific biosynthesis and accumulation of the secondary metabolite, poor yields, risks from pathogens and natural hazards, geographical, seasonal and environmental inabilities to sustain their content and profitable recovery. The medicinal plants in are distributed in natural vegetation as well as cultivated in huge amount in the India (Winter and Tang, 2012).

Primary metabolites

The natural products which participate directly in nutrition and essential metabolic processes (like respiration, storage, cell growth and division, and reproduction) inside the plants are referred to as primary metabolite. The primary metabolites are stored in the big central vacuoles of plant cells. Primary metabolites are used as raw materials for some processes such as fermentation or chemical synthesis. Carbohydrates, amino acid, hormones, nucleotides (Fig. 4), and vitamins are known as primary metabolite. For the regulation of nutritional food quality, colour, taste, odour, and some properties like anticarcinogenic, antiallergic, and decreasing cholesterol level, the primary metabolite are required. The vitamies are essential reqiurenment of living beings for the production of chemicals and there comption depends on the particular condition of the organisms. The plants of family brassicaceae , and horse radish have some bad odur in which glucosinolates ocures. The process like microbial or yeast fermentation plays an important role in producing primary metabolite. The microbes such as *Corynebacterium*, *Bacillus*, and *Aspergillus* have been used for producing primary metabolites, since 1950s. The primary metabolites are always useful, for example: *Acetobacter* bacteria are kind of bacteria which have ability to produce acetic acid. Some microorganisms like *Acetobacter aceti*, *Acetobacter oxydans* and many others are aerobic in nature, while *Clostridium themoacidophilus and Acetobacter xylinum* are anaerobic in nature.

Citric acid plays important role in food industry and used in flavors and beverages. The microorganisms such as *Zymononas mobilis* and *Pseudomonas saccharophilla*, and *yeast* are essential for the ethanol production. The alcohol industries are based on ethanol fermentation (Chen and Wang, 2017).

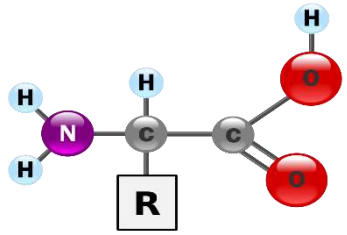

Fig. 1: Amino acid

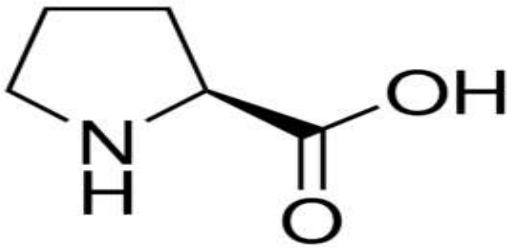

Fig. 2: Proline

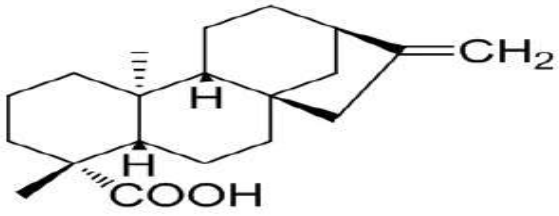

Fig. 3: Kaurenoic acid

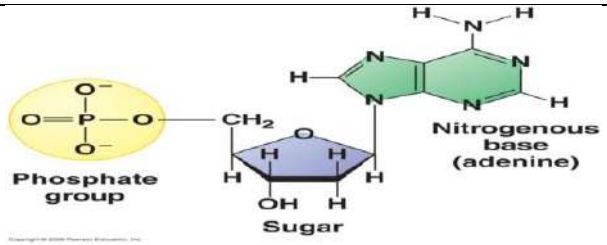

Fig. 4: Nucleotide

Secondary metabolites

The natural plant products that are responsible for influencing ecological interactions between the plant and its environment are referred as secondary metabolites. The Kossel (1891) was first to describe concept of secondary metabolites and also define that these metabolites are different from primary metabolites. Czapek and Fischer Jena (1921) dedicated an entire series of his volume to 'plant biochemistry' named as 'endproduckt'. According to Czapek and Fischer Jena (1921), these products could well derive from nitrogen metabolism, and he referred them as 'secondary modification' such as deamination. These molecules are able to protect plants from pathogens as they have properties such as antibiotic, antifungal and antiviral. The UV-absorbing compounds are present in alfalfa that prevents severe light leaf damage. Alkaloids, phenolic compounds, saponins, and terpenes are some categories of secondary metabolites present in the plants (Meurer-Grimes *et al*., 1992).

Alkaloids

Alkaloids are organic compounds with at least one atom of nitrogen in a heterocyclic ring. No general definition applies to all alkaloids except for the fact that they are all nitrogen-containing compounds. Alkaloids can be classified into various groups based on their basic chemical structure. The basic forms of alkaloids are acridones, aromatics, carbolines, imidazoles, indoles, bisindoles, indolizidines, manzamines, oxindoles, quinolines, phenylisoquinolines, phenylethylamines, piperidines, purines, pyrrolizidines, pyrrolizidines, pyrrolizidines, and pyridines. While plants containing alkaloids have been used as medicines, by humans for at least 3000 years, the activity-responsible compounds were not isolated and characterized until the 19th century. Derivatives of lysergic acid and sulfur-containing alkaloids, such as gliotoxins are detected in fungi. *Lycopodium*, *Ephedra* and *Taxus* alkaloids are recorded for their medicinal use with respect to pteridophytes and gymnosperms alkaloids. There is an unequal distribution of alkaloids between the angiosperms. The examples of alkaloid-rich angiosperms are- members of Chenopodiaceae, Lauraceae, Magnoliaceae, Berberidaceae, Menispermaceae, Ranunculaceae, and Leguminosae. The occurrence of alkaloids in Salicales, Fagales, Cucurbitales and Oleales dicot orders up to the present time, however, is not confirmed (Słomski *et al.*, 2013). Some examples of alkaloids extracted from angiosperm plants are descried below-

Caffeine

A number of botanically distinct species are responsible for caffeine (Fig. 5), such as *Coffea arabia* (Fig. 6), *Camellia sinensis*, *Ilex paraguariensis*, *Paullinia cupana* and *Cola acuminata*. In raw coffee beans, caffeine is attached to chlorogenic acid. The process of roasting releases caffeine and other compounds that contribute to the coffee's fragrance. The body systems like cardiovascular, central nervous system, and respiratory systems get activated by caffeine as it is diuretic in nature.

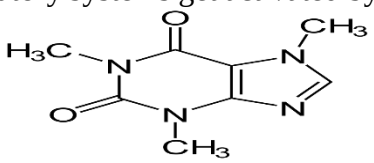

Fig. 5: Structure of the purine alkaloid caffeine from *Coffea Arabica***.**

Fig. 6: *Coffea arabia*

Vinblastine

From *Catharanthus roseus* (Fig. 7), vinblastine is isolated. It has been used as a disinfectant and as a treatment for diabetes and high blood pressure. Vinblastine (Fig. 8), is very critical for cancer fighters as it slowdown the growth of cancerous cells. It is used in the United States and Europe with the other *Vinca* alkaloids such as vinorelbine, vincristine and vindesine, which are in clinical use.

Fig. 7: *Catharanthus roseus*

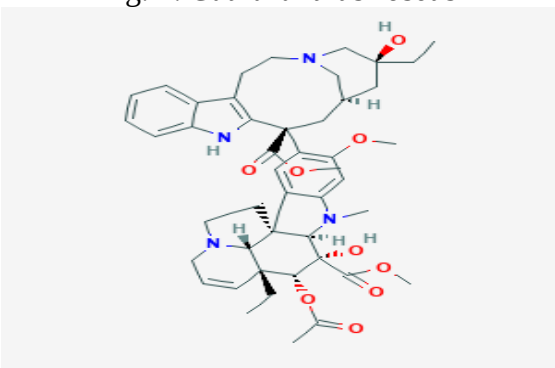

Fig. 8: Structure of the alkaloid vinblastine from *Catharanthus roseus*.

Nicotine

Nicotine (Fig. 9) is found in *Nicotiana tabacum* (Fig. 10), and different species of *Nicotiana*. It has tranquilizing properties and is an addictive tobacco part. It is also highly poisonous, inducing respiratory paralysis at high doses. Nicotine is a cholinergic-receptor agonist of the ganglion with diverse pharmacological actions, including effects mediated by autonomic ganglion, adrenal medulla, neuromuscular junction, and brain binding to receptors.

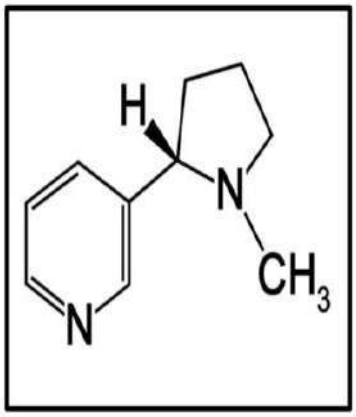

Fig. 9: Structure of nicotine from *Nicotiana tabacum*

Fig. 10: *Nicotiana tabacum*

Phenolic compounds

The most comprehensive group of secondary plant metabolites are potentially phenolics. As a general feature, they share the presence of one or more phenol groups and differ from basic structures with one aromatic ring to highly complex polymeric substances. They are widespread in plants where they significantly contribute to colour, taste and flavour of many herbs, foods and drinks. Many phenolic molecules, especially flavonoids, are effective antioxidants and free radical scavengers. The phenolic compounds are classified on basis of there chemical structures. The simple phenolic compounds, tannins, coumarins, flavonoids, chromones and xanthones, stilbenes, and lignans are known as phenolic compounds (Żak and Kosakowska, 2016).

(i) Simple phenolic componds

The plants have omnipresent phenolic acids; while free phenols are uncommon. Gallic acid is relatively common and is a parent of gallotannins. Gallic Acid (Fig. 11), has many other function in vitro including, antibacterial, antiviral, antifungal, anti-inflammatory, antitumor, antianaphylatic, anti-utagenic, choleretic and bronchodilatory action. Gallic acid is well known for its astringent properties. It also prevents oxidation of insulin and helps to relax the smooth muscles.

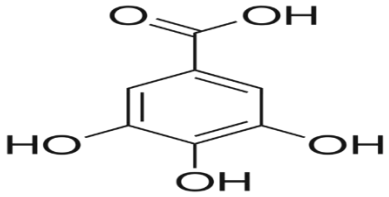

Fig. 11: Structure of gallic acid

The pharmacological activities of several plants are due to simple phenolics, which are attributed to the phenolic content - antimicrobial and diuretic activities. *Capsicum* spp. exhibit circulatory stimulants, rubefacients, and analgesic activities due to the presence of capsaicinoids, which are simple phenolic compounds. Salicylic acid (Fig. 12), Eugenol (Fig. 13), Thymol (Fig. 14), and Hydraquinone are some example of simple phenolic compounds

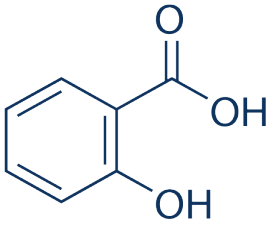

Fig. 12: structure of salicylic acid

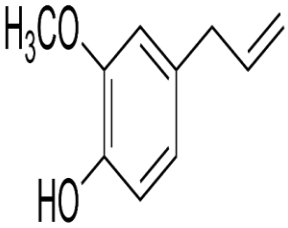

Fig. 13: structure of Eugenol

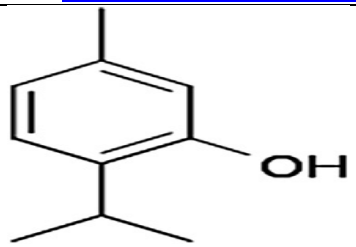

Fig. 14: Structure of Thymol

Tannins

Polyphenols that have the potential to precipitate protein are tannins. For decades, these compounds have been used to turn raw animal hides to leather. Tannin molecules crosslink the protein in this process and make it more resistant to bacterial and fungal attack. However, today, due to their varied composition and biosynthetic origin, many substances considered to be tannins have minimal capacity, to make leather. The Hydrolyzable tannins and condensed tannins are two form of tannins. Tannins that can be hydrolyzed are formed by several molecules of phenolic acids including gallic and hexahydroxydifenic acids that are bound to the central glucose molecule by their ester bonds. Gallotannins and ellagitannins, consisting of gallic acid and ellagic acid units, are the two major forms of hydrolysable tannins. Geraniin (Fig. 15) are isolated from *Geranium robertianum* and *Geranium maculatum* and tellimagrandins are isolated from *Quercus alba* (Oak bark), *Punica granatum* (pomegranate) and *Filipendula ulmaria* are the ellagitannins present in plants of medicinal interest for which structures have been elucidated. The tannins are compounds that are condensed, or proanthocyanidins, based on the arrangement of flavonoid oligomeric precursors, which differ in types of relations between flavonoid units; the patterns of hydroxylation; the stereochemistry of pyranic carbons 2, 3 and 4 and additional substitutes. A variety of medicines (for example, *Camellia sinensis* (tea), *Hamamelis virginiana* leaves and bark) contain condensed and hydrolyzable tannins (Kaufman *et al.*, 2012).

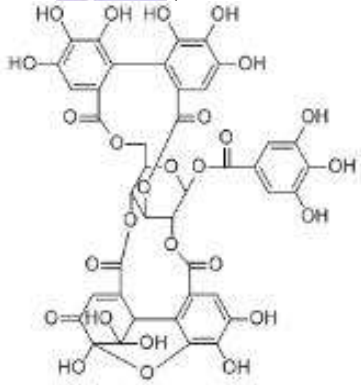

Fig. 15: Structure of Geraniin

(ii) **Flavonoids**

The huge number of natural phenols are flavonoids. Over 2000 substances are now identified, almost 500 in the free state and rest as conjugates. A chroman ring bearing an aromatic ring in position 2, 3 or 4 constitutes the structural skeleton of flavonoids. Flavonoids can be classified according to the central ring's level of oxidation. The plants like *Betula pendula*, *Sambucus nigra*, and *Equisetum ramoissimum* have flavonoids. Flavonal (Fig. 16), flavone (Fig. 16), flavonone (Fig. 16), catechins (Fig. 16), isoflavone (Fig. 16), and anthocyanide (Fig. 16) are examples of flavonoids. The flavonoids have some properties like helps in inhibiting growth of tumor, act as antiallergent and many others (Sturm *et al.*, 2015).

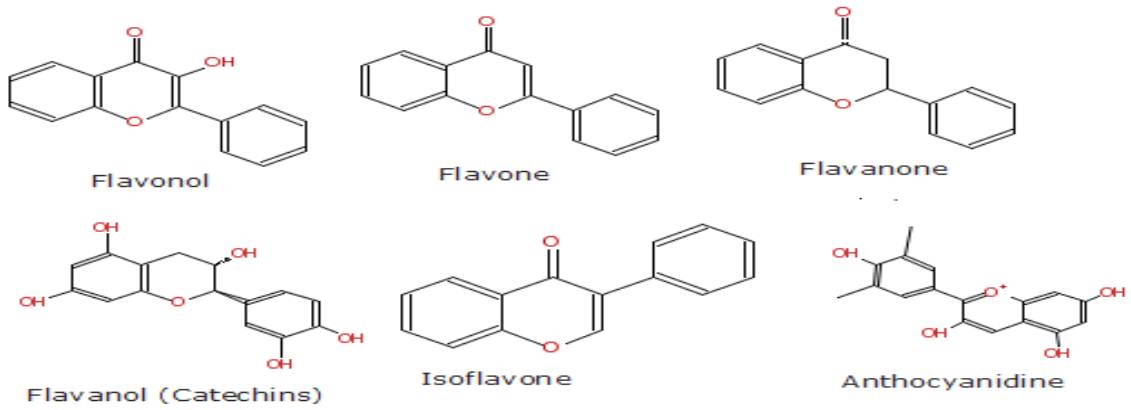

Fig. 16: Structure of flavonol, flavone, flavanone, flavanol, isoflavone, and anthocyanidine.

Saponins

The molecules of saponins can be triterpenoidal (Fig. 17), and steroidal (Fig. 18), and bound to carbohydrate. The acids such as pentose, hexose, and uronic acids are the basic and functional structure of sugar. The Hydrophobic-hydrophilic disproportions within molecules ensure that all these substances act as detergent and have the capacity to reduce surface tension. The genin, or sapogenin, is considered the glycoside part of the saponin compound. The saponins are present in about 500 plants and belong to distinct 90 families and can be extracted from different parts of plant such as flower, stem, leaves, roots, and fruits. The plant species like *Gentiana lutea*, *Panex ginseng* and *Dioscorea villosa* have saponin. The saponin in aqueous solution creates foam and results into in vitro hemolysis of erythrocytes present in blood (Lu *et al.*, 2011).

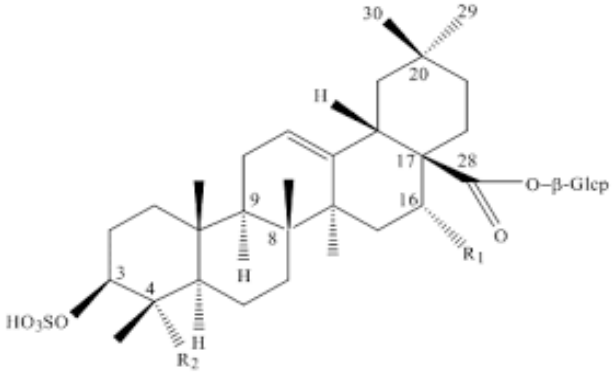

Fig. 17: Structure of triterpenoidal saponin

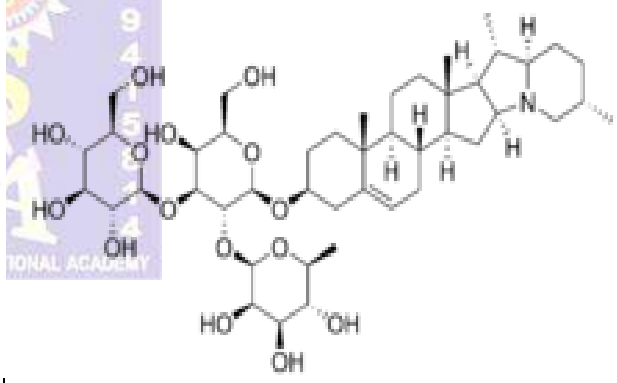

Fig. 18: Structure of steroidal saponin

Terpenes

Terpenes are the largest variety of secondary metabolites present in higher plants. The term ‘terpene’ is coined from word ‘turpentine’ meaning ‘resin’. All the terpenes are made of 5-carbon isoprene molecule units. On the basis of number of isoprene units present in the molecule, the terpenes are classified into hemiterpenes (contain single isoprene molecule), monoterpenes (two isoprene molecule are present) (Fig. 19), sesquiterpenes (three molecules of isoprene) (Fig. 20), diterpenes (four isoprene molecule), sesterterpenes (contains 25 carbons and 5 isoprene molecule), and triterpenes (six isoprene molecule). The great example of terpene is curcumin which is known for its anticancer, antioxidant, anti-inflammatory, diuretic, and astringent properties, (Springs *et al.*, 2011).

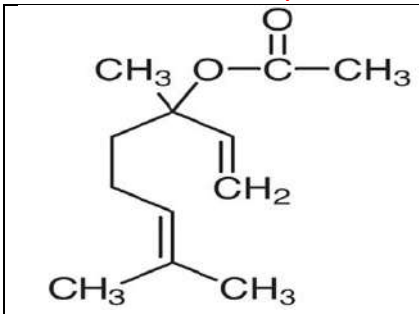

Fig. 19: structure of linalyl acetate (monoterpenes)

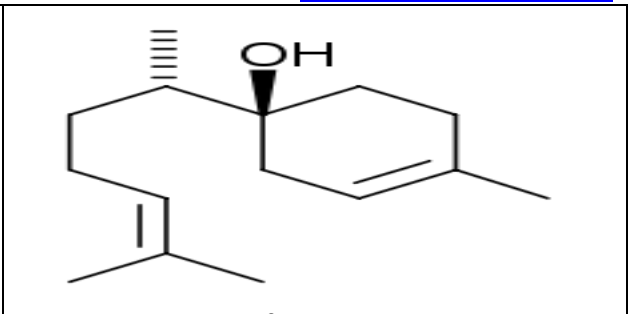

Fig: 20: Structure of bisabolol (sesquiterpenes)

Plant tissue culture (Fig. 21), is carried out by utilizing sterilized explant or tiny pieces of plant tissues which can be obtain from any part of the plant (shoot, root and leaves) and growing them on suitable conditions for the inducing division and growth of cells. Sterilization process plays a very important role in plant tissue culture as it prevents microbial contamination. At the end of the 1960s, the culturing of plant tissue was launched as a technique for the synthesis and recovery of plant secondary metabolites; but due to cells uncertainties like, low outputs and a slow growth problem, metabolites were generated low amounts. In order to synthesize secondary metabolites, the metabolic pathways and the active enzymology of biosynthesis are very important as suggested by Dörnenburg and Knorr (1995). For the production of plant cells, tissues and organs, plant culture uses sterile chemical culture media and incubation in controlled environment. This type of culture has developed into an important method for plant science for studies at various levels. The tissues or cells can be cultured in vitro as callus culture, organ culture, and cell suspension culture. The culture media plays a significant role in plant tissue culture.

Callus culture

The callus culture is raised from cells isolated from explants (part or tissue of plant) under sterile conditions on nutrient medium. It is very necessary to maintain proper ratio of auxin and cytokinin growth regulators in the nutrient medium. To avoid microbial contamination during callus culture, it is important to sterilize the parts of plant by washing and chemical sterilants such as mercuric chloride and sodium hypochlorite. To produce callus, it is important to keep appropriate size of explant. The cells of callus are transferred to the fresh medium. The significance of callus culture is that the plantlets can be regenerated in huge amount just by manipulating by the nutrient composition and hormonal concentration/ratio in the culture medium.

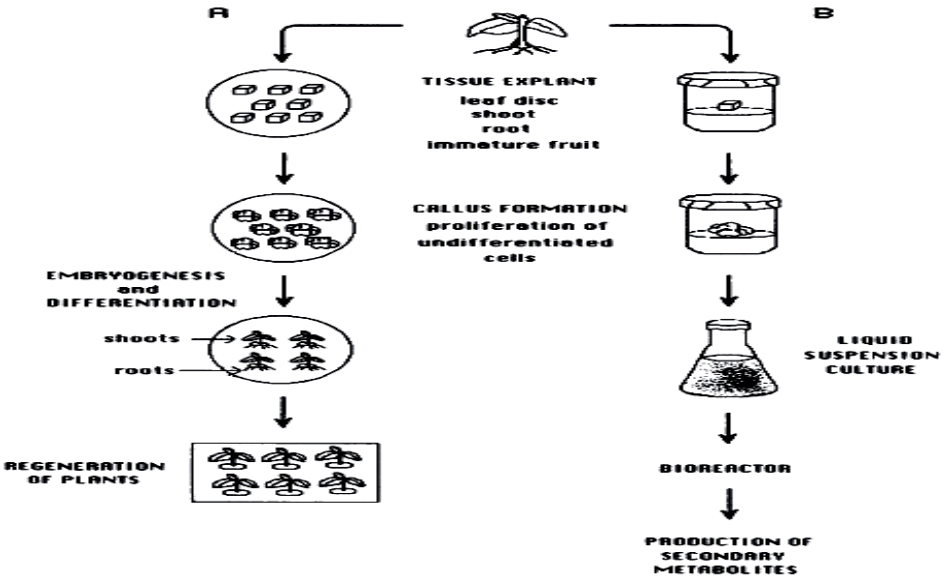

Fig. 21: Production of secondary metabolite through plant tissue culture
Source- www.plantcelltechnology.com

Cell suspension culture

The cells can also be multiplied in cell suspension culture. An excellent opportunity to explore the properties and ability of plant cells is offered by the establishment of single cell cultures. In multicellular organisms, such systems contribute to concept of the inter-relationships and complementary influences of cells. The merits of applying cell cultures over an intact organ or entire plant cultures to synthesize natural products have been recognized by many plant biotechnologists. The shakers are used to perform continuous agitation of cells grown in liquid medium to provide infusion of oxygen and separation of dividing cells. By using mechanical and enzymetic method single cell can be isolated from the plant part, hoever leaves with low vasculature are preferred source. The mechanical method was developed by Gnanam and Kulandaivelu (1969) to isolate mesophyll cells from the dicot and monocot plants leaves. Firstly, the leaves are washed and then ground by pestle and motor method using medium which contains sucrose. Once, the leaf tissues are properly ground and mixed, they are filtered through muslin cloth and placed into centrifugal machine for fractionation and subsequent washing purpose. The free parenchyma cells isolated by mechanical method can be produced in huge amount. In enzymatic method, the tissue from tobacco leaf was treated with pectinase enzyme which resulted hydrolysis of pectin and produced isolated bioactive live cells (Aoki and Takebe, 1969). The yield of active cells can be enhanced by using mixture of enzyme having potassium dextran sulphate. During isolation of cells by enzymatic process, it is seen that the cells of parenchyma are less damaged and by giving osmotic protection to cells, the maximum yield could be achieved. Demerit of enzymatic method is that in case of cereal plants that have elongated formation of their interlocked mesophyll cells, isolation of cells becomes difficult (Lee *et al*., 2016).

Callus can be developed continuously in the favourable culture medium with stable cell division through repeated sub-culturing.

The secondary metabolites such as phyllantine and hypophyllanthin extracted from plant *Phyllantus amarus* has great medicinal value and used as hepatoprotective drug. The alkaloids are present in the seed of plant *Phyllantus amarus* in huge amount. For the extraction of phyllantine and hypophyllanthin, their seeds are used as explant and cut into the size of 1cm and then placed into half strength MS culture medium for in vitro germination. Callus produced in in vitro cultures is used for raising cell suspension culture as shown in (Fig. 22). The soft form of callus can be utilized for the establishing fresh cell suspensions in liquid media.

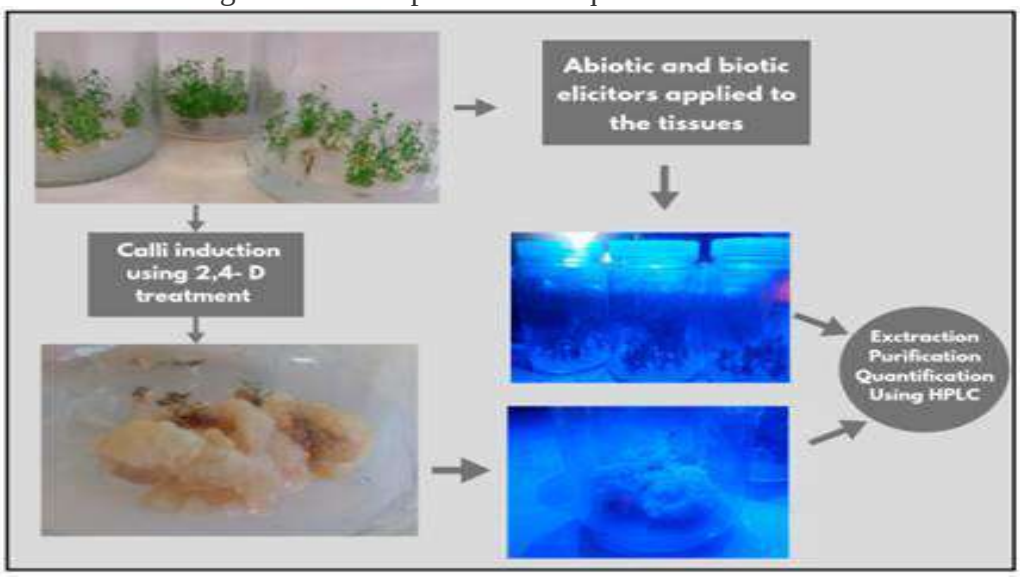

Fig. 22: The in vitro culture is used to produce phyllanthin and hypophyllanthin from *Phyllanthus amarus*.
Source- www.scielo.br.com

The MS medium is also used to culture the cells of plant *Hypericum perforatum* on semi-solid medium using agar as gelling agent. The stems of *Hypericum perforatum* are used as explant because they are able to generate friable callus. Stem is washed under tap water and surface sterilized by using 75% of ethanol for approx. 60 seconds. Subsequently the explant is immersed in the 10% solution of NaOCl 30 seconds and distill water is utilized for rinsing purpose. After rinsing, it is sliced into 5 mm fragments. Growth regulators such as 2,4- Dichlorophenoxy acetic acid, benzyladenine, and carbon source (sucrose) are supplied to the culture medium to initiate the development of callus. The friable callus is transferred to flask containing liquid medium and placed on shaker for 20 days' culture passage. After 20 to 25 days of in vitro culture, it is observed that cell suspensions are rich in flavonoids due to utilized elicitors such as jasmonic acid and salicylic acid (Wang *et al*., 2015).

The hairy root culture is the most capable and durable method of plant cell culture to produce stable biochemical, maintained cell variability and can generate variety of secondary metabolites having medicinal properties. The bacteria such as *Agrobacterium rhizogenes* is used to produce hairy root culture.

In *Artemisia vulgaris*, the *Agrobacteria rhizogenes* strain A4GUS is used for the transformation of shoot apex, leaves, and nodes and the cells of leaves show great successes of transformation up to 92.6% (Sujatha *et al*., 2013). The secondary metabolites such as Ajmalicine (Fig. 23) with great medicinal importance (in diseases related to the circulatory system) are produced from *Catharanthus roseus* by using hairy root culture (Thakore *et al.*, 2017).

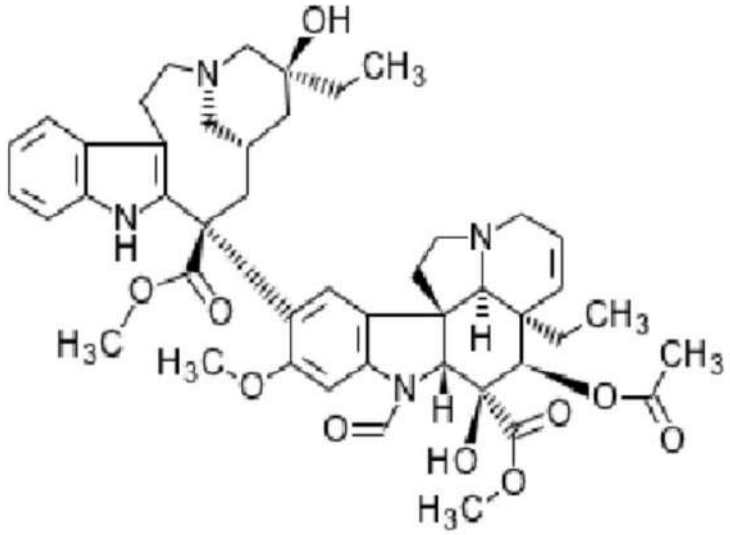

Fig. 23: structure of Ajmalicine Source- www.pubchem.ncbi.nlm.nih.gov

Disadvantage of producing secondary metabolite by in vitro culture methods are:

- Reduced yield of biomass due to which the plant having medicinal importance cannot be produce in large scale.
- The in vitro methods are very expensive and uses expensive methods such as-

(i) The extraction of secondary metabolite especially used as drug,

(ii) Their purification and analysis costs about 100 million dollars as they use method like high-performance liquid chromatography.

By using bioreactors, secondary metabolite can be produced in large amount with cost effectiveness. The in vitro production of secondary metabolites also requires skilled workers with furnished and developed laboratory under sterilized condition to avoid the interference of unwanted toxic components (Chandran *et al*., 2020). Despite this, secondary metabolites for industrial purposes are very likely to be produced using tissue culture. Some strategies are developed for the purpose of high biomass accumulation and efficient biosynthesis of secondary

metabolites, including the improvement in optimization of the medium and cultivation environments, elicitation of secondary metabolites, nutrient and precursor feed, immobilization, permeabilization, and biotransformation techniques.

Medium optimization

The biomass accumulation and biosynthesis of secondary metabolites in the plant cell culture is influenced by numerous physical and chemical factors. The crucial and basic factor that affects the cell metabolism and physiology is the composition of a medium. The Variables such as the suitable salt strength, sugar, nitrate, phosphate as well as growth regulator levels in the medium are main factors. There are various types of culture media having different media formulations examined and utilized to grow cell or tissue suspension cultures for secondary metabolite production. Some extensively used media are Murashige and Skoog (MS), Gamborg's (B5), Schenk and Hildebrandt (SH) and Linsmaier and Skoog (LS). The B5 differs from MS medium due to difference in the amount of nitrogen in the form of ammonium. For the growth of the isolated cells and tissue, concentration of medium components (salt strength) is essential. In *Gymnema sylvestre* the full strength MS medium is used in cell suspension culture medium and suitable for both biomass and gymnemic acid accumulation as shown in (Fig. 24) (Murthy *et al*., 2014).

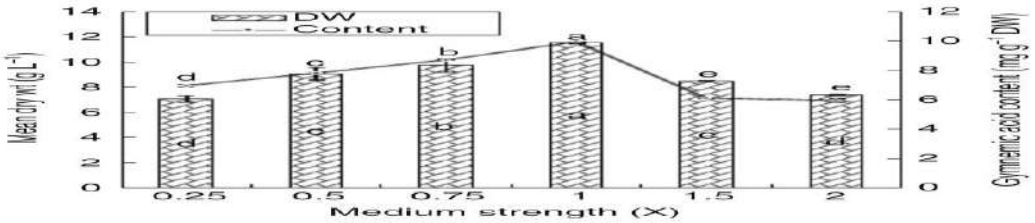

Fig. 24: Impact of medium strength on biomass accumulation and development of gymnemic acid.
Source- www.researchgate.net.com

The accumulation of biomass and secondary metabolites in cell and organ cultures is influenced by environmental factors such as light temperature, medium pH and gases. The temperature range of 17-25^{0}C are used for the nurture of cultured cells and tissues. In cultured tissues and cells, the accumulation and production of secondary metabolites is also influenced by light. In *Perilla frutescens*, Zhong *et al.* (1991) has signified the consequences of light-quality, strength and time for the irradiation of cell growth and anthocyanin pigment. The most significant criteria regulated by flask-scale to large-scale bioreactor cultures are agitation. The increased mass transfer and uptake of nutrients by cells from the liquid and gaseous phases promotes better growth and occurs as a result of crop mixing. The important factor controlled in bioreactor cultures for culture process optimization is aeration after agitation. The three main functions, such as the maintenance of aerobic conditions, desorption of volatile products and elimination of metabolic heat through mixing and air flow, are maintained by aeration. In ginseng culture, it is observed that oxygen supply affects secondary metabolite production (Fig. 25) (Murthy *et al.*, 2014).

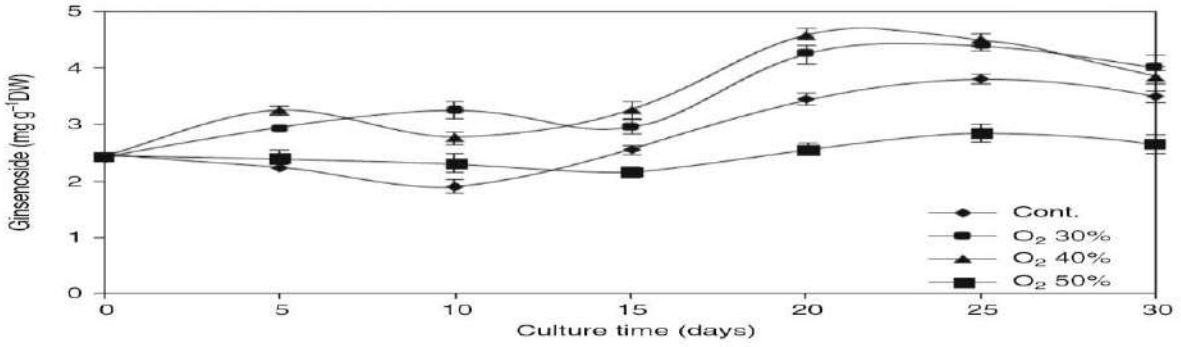

Fig. 25: Kinetics of the synthesis of *Ginseng saponin* by bioreactor-cultured *Panax ginseng* cells.
Source- www.researchgate.net.com

Elicitation

The various kinds of stress are responsible for the production and accumulation of secondary metabolites in plant cells and known as elicitors. The elicitors have also been used for enhancing the secondary metabolite production. On the basis of origin, elicitors can be distinguished into biotic or abiotic. The stress such as insect or pathogen attack are referred as biotic stresses and temperature, salinity, water stress, radiation stress, heavy metal and mineral stress are abiotic stresses. Biotic elicitors may be either exogenous or endogenous, and come from the biological base. Exogenous biotic elicitors are microbial cell wall components such as chitosan, chitin, or plant cell wall components such as polysaccharides or oligosaccharides. Some signaling molecules such as polysaccharides or oligosaccharides are planned for elicitation routes because they induce plant defense responses like the disease invasion (Liang *et al.*, 2018). Polysaccharides (released by the deterioration of the cell wall from pathogenic substances) and ligands or proteins such as salicylic acid or plant-built methyl jasmonate are endogenous biotic elicitors. Specific cell membrane receptors have tendency to recognize biotic electors and transfer the stimulus by the signal transduction into the cell, leading to phytoalexin development. The elicitors are used to enhance the production of secondary metabolite in cell or tissue cultures. The fungus, bacteria and yeast have ability to work as elicitor, and polysaccharides, glycoproteins, inactivated enzymes, distilled crudlan, xanthan and chitosan salts and heavy metals are responsible for enhanced development of secondary metabolites. The methyl jasmonate and salicylic acid are signaling molecules used for the accumulation of secondary metabolites in the cell cultures. Elicitor treatment is an important process for the successful production of secondary metabolites which include elicitor concentration, period of exposure, culture age and physiological stage. The signalling components that result in an enhanced development of secondary metabolites such as flavonoids, alkaloids, terpenoids and phenylpropanoides have been reportedly phytohormones including salicylic acid and jasmonate made in plants to repair stress or pathogens invasion. Yu *et al.* (2002) have investigated the impact of jasmonic acid on ginseng-adventitious roots. The increased jasmonic acid concentration contributed to decrease of both fresh and dry biomass, but the amount of ginsenoside increases upto 5.2 fold (Akula and Ravishankar, 2011). The Biomass reduction has been addressed through two step strategies, i.e. (i) for 25 days the adventitious roots are grown without any elicitor and then (ii) jasmonic acid is added and total ginsenosides and Rb group of ginsenosides are increases by 5 to 5.6 folds respectively (Chodisetti *et al.*, 2013).

Bacterial elicitors are also used to improve the synthesis of secondary metabolites. Gram-positive and Gram-negative bacterial strains were used in *Scopolia parviflora* adventitious hairy root cultures as elicitors of tropane alkaloid biosynthesis (Jung *et al.*, 2003). Treatment with *Rhizobacterium* elicitor leads to the slow growth in *Hypericum perforatum* from which hypericin and pseudohypericin are derived (Manero *et al.*, 2012). Few elicitors have been used to examine the ability of callus mass for the production of phyllantine and hypophyllanthin (Kapoor *et al.*, 2018).

Nutrient feeding

Nutrient feeding is another approach to enhance the secondary metabolite production. During the ginseng adventitious root culture, various nutrients of culture medium are exhausted by the end of culturing for 40 days. Jeong *et al.* (2008) replenished the cultures with 0.75 and 1.0 strength media after 10 and 20 days of cultivation, a 27.45% increase in dry biomass (28.66 g L^{-1} with replenishment treatment) and 8.25% increase in ginsenoside content (4.93 g g^{-1} DW) were shown by cultures replenished with fresh medium (1.0-strength MS medium after 20 days of culture). A sugar feeding method is designed on the basis cell growth effected by sucrose, and is used to enhance the production of saponin derived from *Panax notoginseng* (Zhang, 1996, Zhang *et al.*, 1996).

The biggest drawback of batch processes is that the sterilization, filling, emptying and cleaning of the machine and media takes a considerable amount of time. It is possible to improve the cost

efficiency of cultured plant cells through various operating modes. These operational modes include fed-batch, repeated fed-batch, semi-continuous and ongoing cultivation developed by biochemical engineers. The operation of the fed batch involves the continuous or intermittent addition of one or more nutrients to the initial medium after the initiation of cultivation or at the batch phase level. Unchecked feedback variants (for example in chemostats, substrates are constantly being fed) and feedback control in continuous cultivation are also used in trubidostats, the cloudiness of culture is sustained by calibrating the rate substrate feeding and auxostats, the ethics of dissolved oxygen in the medium is fixed. In perfusion cultivation continuously fresh medium is fed to the bioreactor and cell free medium are removed constantly (Wu *et al.*, 2009).

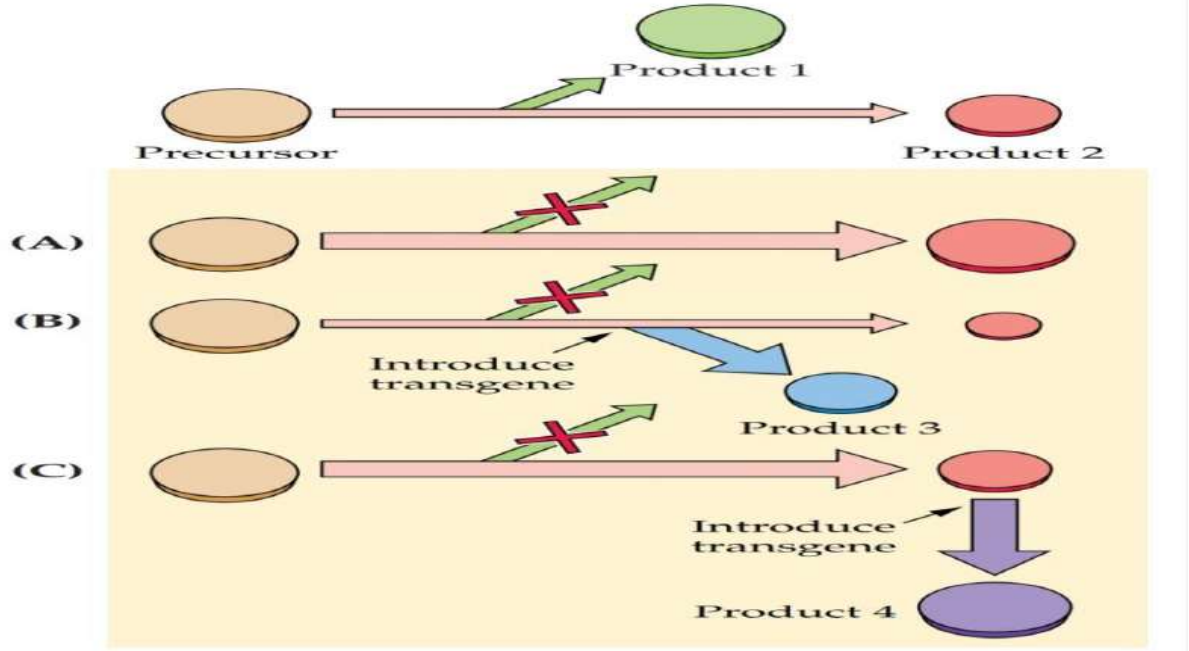

Fig. 26: Using antisense or overexpression, medicinal plants can be tailored to produce pharmaceutically important alkaloids by eliminating interfering metabolic steps or by introducing desired metabolic steps. Expressing an entire alkaloid biosynthesis pathway of 20 to 30 enzymes in a single microorganism is currently beyond our technical capability. However, altering the pathway in a plant and producing the desired alkaloid either in culture or in the field may now be possible. For example, to accumulate more of the end product alkaloid, a side pathway that also uses the same precursor may have to be blocked (A). To accumulate an alkaloid not normally produced in a particular plant species, a transgene (from another plant or a microorganism) may be introduced (B). If the end product alkaloid would be more useful as a particular derivative, for example, as a more soluble glycoside, a gene that encodes a glycosyl transferase could be introduced (C). Source- www.sciencedirect.com

Precursor feeding

The precursors are converted into products by utilizing their enzyme systems under plant cell culture. The production of secologanin and indole alkaloids (Fig. 26) gets enhanced by the addition of loganin, tryptophan and tryptamine in the *Catharanthus roseus* suspension cultures. When it is used for the cell culture medium, factors such as the concentration of the precursor and time of addition are considered. The accumulation of paclitaxel in *Taxus cuspidata* is improved by feeding phenylalanine and feeding of cholesterol influenced the production of conessine in *Holarrhena antidysenterica* cell cultures (Wang *et al.*, 2001).

Permeabilization

The purification process becomes easier if plant secondary metabolites formed by the plant cell cultures are generally stored in the vacuoles, and therefore extraction of desired product into the culture medium and continuous recovery and production of the product is carried easily. The possibility of product inhibition reduces with the removal of secondary metabolite from vacuoles of the plant cells and in result productivity increases. Some organic solvents used are permeabilizing agents such as dimethylsulfoxide (DMSO) and polysaccharides like chitoson, and

isopropanol. These permeabilizing agents make plant cell membrane permeable in a reversible manner. These permeabilizing agents also affect the cell viability. For example, hexadecane, decanol and dibutyl phthalate are used for permeabilization of paclitaxol in *Taxus chinensis*. The methods such as electric field stress and ultrasound are also used for permeabilization. The substance having power of permeabilization should be chosen in light of their effect on cell growth and the release of secondary metabolites should be considered (Li *et al.*, 2001).

Immobilization

The problem of low shear resistance and the tendency of cell aggregation are rescued by immobilization of plant cells with the suitable matrix. The gel entrapment and surface immobilization are two methods used for cell immobilization. The immobilization is a technique in which entrapment of cells in a specific gel are involved. The matrix used for entrapment should not be toxic to the cell and must have better polymerization activity and should not be expensive. As a, entrapment matrix, there is use of agar, agarose, gelatin, carrageenan, polyacrylamide and calcium alginate. Brodelius *et al.* (1979) was the first who reported immobilization in *Catharanthus roseus*, *Morinda citrifolia* and *Digitalis purpurea* culture.

The merits of immobilization include:

- the possible maintenance of biomass over the prolonged period time,
- the viability of cell is extended in stationary stage,
- simplified downstream processing, in small bioreactors produce high cell density with cheaper cost and reduced risk of contamination,
- shear stress is reduced, minimization of fluid viscosity in cell suspensions results in mixing and aeration problem,
- accumulation of product is increased.

However, few times it is observed that the immobilization of cell shows dramatic effects for the production of secondary metabolites in the plant cell culture, for example capsaicin production shows 100-fold increment through immobilized cells with foam and gel, and *Coffea arabica* and *Catharanthus roseus*, show 3-4-fold increment in methylxanthin and ajamalicine accumulation, respectively from the gel immobilized cells. Immobilization is an identified strategy for increasing bioproduction of secondary metabolites in plant cells (Dörnenburg, 2004).

Two-phase system

The absence of an important enzyme involved in the synthesis of secondary metabolite is not always responsible for low level of accumulation of secondary metabolites, the assessment of enzyme or non-enzyme degradation of the medium may be due to this product. A dividing technique should be developed to concentrate the product. For in situ product separation of crops of plant cells, plant cells that consist of an alkaline process of nutrients and heavy polar biosorbents, have a liquid-solid cultivation system (two phase systems) preferable, because many plant cells are supposed to be polar in nature and weakly bind in the lipophilic stage of liquid-liquid systems. To separate secondary metabolite successfully in the cell suspension cultures of several systems. Some adsorbents examined and used are activated charcoal, RP-8(lipophilic carrier), Zeolith, xad-2, xad-4, xad-7 (XAD is a neutral resin and ion exchanger), polyethelene glycol and wofatite, polydimethylsiloxan. The Uptake and oversupply of paclitaxol from *Taxus* suspension cultures, anthraquinones from *Rubia akane* suspension cultures, triptolide from adventitious root cultures of *Tripterygium wilfordidi* can be effectively performed by Ambrilte XAD-7 (Miao *et al.*, 2013).

Biotransformation

The process of transformation of stereospecific and regio-selective chemicals is stimulated by entrapped enzymes or biological systems or permeabilized cells. The production of high value metabolites through plant cell cultures is followed by biotransformation. During biotransformation process, some reactions such as hydrolysis, glycosylation, glucosylation, hydroxylation, methylation, oxidoreduction, acetylations, isomerization and esterification of various substrates

are involved during biotransformation. There is high biochemical potential for the production of specific secondary metabolites in plant cell cultures but desired products are not accumulated due to certain metabolic reasons. The possibility of transforming exogenous substrate into desired products can be obtained by cell cultures and the industrial byproduct can be transformed into expensive product by plant cell culture and enzymes. The free suspended and immobilized cells of *Capsicum frutescens* were used for the conversion of protocatechic aldehyde and caffeic acids in vanillin and capcininin (Rao and Ravishankar, 2000).

Bioreactor

The cells present in plant cell cultures have unusual characteristics such as lower stability of productivity, higher shear sensitivity, sluggish growth rate, and low oxygen requirements. A wide number of bioreactor (Fig. 27) designs have been tested and used for plant cell suspension cultures. With some modifications to microbial culture systems, stirred tank reactors, airlift reactors, and bubble column reactors for plant cell cultivation are simply extensions and the world's largest plant cell culture facility installed in Germany (Georgiev *et al*., 2009). The Centrifugal impeller bioreactor is based on the principle that Wang and Zhong (1996) have developed a centrifugal pump, especially for shear-sensitive systems such as high-shear-sensitivity cultured plant cells. In stirred tank reactors equipped with a centrifugal impeller for the production of azadirachtin, effective scaling of *Azadirachta indica* suspension cultures has been established. In a novel centrifugal impeller bioreactor (CIB) in which initial k_L a was identified as a key factor affecting cell growth and production of *Ginseng saponin* and polysaccharide, a scale-up of high-density *Panax notoginseng* cell culture was demonstrated. Based on the initial level of k_La, the CIB high-cell-density cultivation method was successfully scaled up from 3 to 30 L in the laboratory. The maximum dry cell weight (DW) and production titer of *Ginseng saponin* and polysaccharide in the 30-L CIB reached 25.5, 1.7 and 2.9 g L^{-1} (on day 15) at baseline k_La and 28.7 h^{-1} respectively. The advantages and disadvantages of few standard bioreactor systems and airlift bioreactors seemed to be optimal, outlined by Dörnenburg and Knorr (1995). In addition, airlift bioreactors that disperse the air by sparger from the base of the reactor are sufficient for the cultivation of hairy roots and adventitious roots of different medicinal plants (Pan *et al.*, 2000).

Fig. 27: To cultivate the adventitious roots of ginseng, large-scale (10,000l) bioreactors were created. Source-www.merkmillipore.com

Stage 1 – Biomass accumulation
1. Selection of efficient cell lines or clones
2. Medium optimization
(a) Selection of suitable medium and salt strength
(b) Carbohydrate source and concentration
(c) Nitrate levels
(d) Phosphate levels
(e) Growth regulator levels
3. Inoculum size
4. Optimization of the cultural environment
(a) Temperature
(b) Illumination
(c) Quality of light or combination of lights
(d) Medium pH
(e) Aeration and agitation
State 2 – Accumulation of bioactive compounds
5. Elicitation
6. Nutrient feeding
7. Precursor feeding
8. Permeabilization
9. Immobilization
10. Two phase system
11. Biotransformation
12. Organ cultures
13. Large-scale cultures

Fig. 28: Strategies to improve the production in plant cell and organ culture of secondary metabolites.
Source- www.researchgate.net.com

Strategies to enhance the in vitro production of secondary metabolites

The important factors in the commercial production of secondary metabolites are steady performance and high yields. In several cases, the process of accumulation of biomass and biosynthesis of secondary metabolites through plant tissue cultures takes place in two stages:
(1) Involvement of cultured tissue in biomass growth, multiplication and accumulation at the initial stage, and
(2) Synthesis of accumulated cell metabolites at the later stage.
It is quite possible to achieve high yield of enhanced metabolite production by following two steps process that is focused strategy for the accumulation of biomass and using strategies for the stimulation of biosynthesis of metabolites (Fig. 28) (Nagella and Murthy, 2011). To upgrade the production and productivity of secondary metabolites, some approaches have been introduced such as traditional and metabolic engineering strategies.

(i) Traditional Strategies

Secondary metabolites are formed from intermediates or end products of primary metabolism and are thus an output of primary metabolism. It depends on the conditions at which substrates are redirected from main metabolic pathways to secondary biosynthetic pathways. Their synthesis relies on both biotic and abiotic variables, such as growth and physiology, temperature, humidity, strength of light, etc. The metabolite productivity of in vitro cultures depends on the composition of culture media, pH, density of inoculum, temperature, light density, agitation, aeration, and many others. Such factors must therefore be optimized to maximize growth and metabolite productivity. The choice of appropriate cultural media plays a crucial role in the development of secondary metabolites. The metabolite synthesis is regulated by the components of the culture medium, including macro- and micronutrients, vitamins, sugars, amino acids, and plant growth regulators

such as cytokinins, auxins, gibberellins, jasmonates and salicylate. Microbes have been documented to activate several secondary metabolic pathways. There has been an interest in the use of bacterial, fungal and yeast extracts to activate secondary metabolites. The very powerful technique to improve the efficiency of secondary metabolites in plant tissue culture is the use of fungal elicitors. It induces the expression of unique plant genes, thereby increasing secondary metabolites by activating secondary metabolic pathways. In the process of physiological progression, it induces plant phytoalexins to prevent plant disease. Some fungus such as *Fusarium*, *Pythium*, Yeast, *Aspergillus*, *PenicilIium*, and *Trichoderma* are indeed harvested as fungal elicitors and used in medical plants to cause secondary metabolites (Cardoso *et al.*, 2019).

(ii) Metabolic engineering

Metabolic engineering is the alteration of an organism's metabolic pathways using modern biological techniques such as genomics and proteomics, metabolomics, to obtain commercially relevant metabolites. By trying to divert precursors, enzymes, regulatory proteins with the aid of recombinant DNA technology, strategies include over expression or down regulation of metabolic pathways. Plants contain multiple metabolic pathways responsible for complex metabolites' biosynthesis (DellaPenna, 2001). For overproduction and isolation of economically relevant pant metabolites, these pathways can be reconstituted in heterologous hosts. The path of terpenoids is also known as the path of isoprenes. In plants, terpenoid biosynthesis uses two independent pathways: the pathway of mevalonic acid (MVA) and the pathway of methyl-D-erythritol (MEP). Precursors for the synthesis of brassinosteroids, sesquiterpenoids, phytosterols, triterpenoids, and polyprenols are served by the MVA pathway. In fatty acid biosynthesis, the polyketid pathway plays a critical role. It is derived either from acetyl CoA or from malonyl CoA. Acetogenins, Jasmonate 6-methylsalicylic acid, plumbagine, coniine and anthraquinones are the secondary metabolites formed by polyketide pathway (Xu *et al.*, 2015).

Up-regulating Pathways

Transcription factors are the proteins that bind to specific locations such as DNA promoter or enhancer to regulate transcription, or target gene expression. They encode genome data and modulate transcription rates. The activator domain and the DNA binding domain are two effective domains. The DNA binding domain has amino acids that describe the particular DNA base on the regulatory element, while the activator domain has binding sites for other proteins called activation and trans-activation functions (transcription co-regulators). For secondary metabolite development, multiple transcription factors have been identified. MYB transcription factors in several species of plants have been identified as controllers of several biosynthetic routes (Dubos *et al.*, 2010). The Transcription factors are able to regulate anthocyanin accumulation as well as flavonoids in plant species such as *Arabidopsis, Petunia hybrida, Solanum lycopersicum, and Ipomea*, and R2R3-MYB, WD repeat (beta-transducin repeat), elementary helix-loop-helix genes, adenine nucleotide translocator and adenine nucleotide translocase-1, APETALA2 or ethylene are response factor and (AP2/ERF), WRKY, NAC, SQUAMOSA are Promoter Binding Protein perform transcription. A transcription factor that acts on particular pathway genes is the challenging factor in up- regulating pathways. An approach to this challenge is to generate synthetic transcription factors that target more than one essential gene (Yu *et al.*, 2012).

Down-regulating Pathway

The enzyme level, increasing catabolism and carbon flux into competitive pathways will decrease the synthesis of some metabolites. Unwanted metabolites can be prevented by hiding genes which up-regulate or increase the metabolism. Modern r-DNA technology such as antisense RNA, interference with RNAi can be used to phase out undesired metabolites and co-suppression. It has reportedly been used to manipulate secondary plant metabolites such as phenylpropanoids, alkaloids and terpenoids (Wagner et al., 2008). The phenyl-propanoid pathway modulation using RNAi has led to a considerable coloring in flowers, low pigment seeds. The production of codeine and morphine has been increased in opium pappies, cyanogenic glucosides have been lowered in

cassava, flavonoids and carotenoids have increased, and fragrances have been controlled in petunia flowers (Gómez-Galera *et al.*, 2007).

(iii) The Nanoparticale Technique

The particles are classified as nanoparticles within the size range of 1–100 nm. These particles have been used to promote germination quality, promote plant growth, and enhance bioactive metabolites in plant tissue culture (Wang *et al.*, 2016).

In order to enhance secondary metabolite production in plants, the effects of certain important metal oxide nanoparticles such as titanium oxide, zinc oxide, iron oxide and copper oxide are recognized. Titanium oxide naoparticles with a large increase in *Cicer arietinum* embryonic calli include gallic acid, chlorogenic acid, o-coumaric acid, tannic acid and cinnamic acid (Mohammed *et al*., 2015). The Artemisinine content concentration was increased in *Artemisia annua* with silver nanoparticles therapy (Zhang *et al*., 2013). The silver nanoparticles are also responsible for increased concentration of diosgenin in fenugreek (Jasim *et al*., 2017) and production of anthocyanin and flavonoid in *Arabidopsis thaliana* (Garcia-Sanchez *et al*., 2015). Cadmium oxide nanoparticles have developed an increased concentration of ferulic acid and isovitexin in barley plants (Ruttkay-Nedecky *et al.*, 2017).

(iv) The selection of cell lines and clone

The strategy of selection of high yielding cell lines and clones plays a crucial role in the production of secondary metabolites from plant cell culture at industrial scale. In order to obtain high yielding cell lines, the culture explant process is initiated by the selection of parent plants containing a high amount of the desired callus induction product. The accumulation of secondary metabolite in plant is the genotype. During secondary metabolite production at various levels, inter- and intra-differences occurs in cell lines which attribute to phenotypical variations or somaclonal variations. The phenotypical variation produces unstable high yielding cell lines and occurs due to cellular differentiation and on the exposure of numerous plant growth hormones get expand while somaclonal variation produce stable high yielding cell lines. The camptothecin (a quinolone alkaloid used as anticancer drug) present in different plant species with variable amount is the suitable example for proving secondary metabolite is genotype. For the production of desired compounds, it is very important to select a suitable genotype and tissue. After selection, isolation of cell or tissue lines for growth and secondary metabolites accumulation is necessary. If the required products are pigmented, then the selection of cell lines and clones are done by visual screening. The enhanced production of product known as anthocyanin through clonal selection and visual screening has been observed in *Euphorbia milli* and *Daucus carota* (Yamamoto *et al*., 1982). The analysis of growth of the cell lines or root clones (adventitious or hairy roots) in suspension culture of required product plays a major role in the selection of cell line and clone which is considered as superior to visual selection technique. The rosmarinic acid is found in *Orthosiphon stamineus* present in two cell lines which are selected and identified and produced in high amount under cell suspension culture (Liang *et al*., 2006).

Conclusions

The plant cell and tissue cultures are felicitous techniques for producing valuable secondary metabolites of economic importance and used in pharmaceutical, food, fragrance, colour, and textile industries etc. The advanced biotechnology approaches such as signal transduction and engineering for highly induced biosynthesis of interested products (metabolites) make this technology very fascinating. Plant cell cultures give rise to improved biomass accumulation and secondary metabolite production by increment in folds. The composition of culture medium plays an important role in the selection of culture media for culturing plant cells. At bioreactors level, parameters like agitation and aeration, elicitation, nutrient feeding, precursor feeding, permeabilization, and immobilization are important in determining the synthesis, accumulation and release of secondary metabolites and must be optimized properly with each production system. The enzymes present in cultured cells catalyze the biotransformation of low valued product into

high valued product and further make in vitro production of secondary metabolite production as novel and attractive source. The chemicals used in permeabilization to induce extracellular release of product should be non-toxic and cheap. In vitro approach has great potential for a broad range of applications, ranging from improving the production of secondary metabolite to the introduction of new pathways in plant. Traditional and Metabolic engineering strategies are introduced to accelerate the production and productivity of plant secondary metabolite. It is important to expand the knowledge about plant secondary metabolism, at the level of the intermediates, the enzymes and genes for developing full potential of metabolic engineering. The Proper understanding and rigorous analysis of these strategies can leads towards successful commercialization of plant cell bioprocesses.

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